



TITLE:

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Acquired resistance of leukemic cells to AraC is associated with the upregulation of
aldehyde dehydrogenase 1 family member A2

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Abbreviation: AraC, 1- β -D-arabinofuranosylcytosine; AML, acute myeloid leukemia;

ALDH1A2, aldehyde dehydrogenase 1 family member A2.

Key words: drug resistance, AraC, ALDH1A2

Abstract

The elucidation of drug resistance mechanisms is very important in the development of clinical therapies for the treatment of leukemia. To study the drug resistance mechanisms, protein expression profiles of 1- β -D-arabinofuranosylcytosine (AraC) sensitive K562 (K562S) cells and AraC resistant K562 (K562AC) cells were compared using two-dimensional fluorescence difference gel electrophoresis. In comparison of protein expression profiles, 2073 protein spots were found to be altered, 15 proteins of them were remarkably altered. These proteins were identified by mass spectrometry. The most differently expressed proteins were aldehyde dehydrogenase 1 family member A2 (ALDH1A2) and vimentin. Both proteins were verified using reverse transcriptase polymerase chain reaction (RT-PCR) and Western blot analysis. ALDH1A2 protein was found to be effective in AraC resistance. ALDH1A2 knock down (KD) induced sensitivity to AraC treatment in K562AC cells, and ALDH1A2 overexpressed K562S cells acquired the AraC resistance. Further, the findings also suggest that ALDH1A2 expression is increased after the appearance of AraC resistance in clinical cases. These results will be helpful in understanding the mechanism of AraC resistance.

Introduction

Chemotherapy has been very useful in the treatment of leukemia. However, cellular drug resistance is the greatest impediment to successful treatment. The mechanism of drug resistance can roughly be divided into two processes: drug uptake or metabolism, and inhibition of apoptotic cell death or increased repair of drug-induced DNA damage [1, 2].

Cytosine arabinoside (1- β -D-arabinofuranosylcytosine, AraC) is one of the most effective drugs in the chemotherapeutic treatment of acute myeloid leukemia (AML) [3]. AraC is also active against other hematologic malignancies such as acute lymphoblastic leukemia (ALL) and non-Hodgkin's lymphoma [4]. Laboratory investigations to elucidate the biochemical correlation between drug action and drug resistance mechanisms have extensively examined the clinical importance of AraC, in order to obtain leads for the development of better therapeutic strategies.

AraC is converted into its active metabolite, cytosine arabinoside triphosphate (Ara-CTP), intracellularly in leukemic blasts by deoxycytidine kinase (dCK) [5]. Ara-CTP is generally considered to be the active form of the drug since it both inhibits DNA polymerase and leads to AraC incorporation into DNA [6]. AraC is catabolized to

the non-toxic metabolite, arabinosideuridine (AraU), by a rapid deamination mediated by cytidine deaminase (CDD) [7, 8]. Various biochemical mechanisms of AraC resistance have been shown in experimental animal tumor models or in vitro systems [9]. Low AraC phosphorylation or low dCK in marrow blasts has been associated with poor response [10]. It has also been reported that AraC resistance is induced when the influx of AraC is reduced because of the human equilibrative nucleoside transporter 1 (hENT1) [11], when the degradation of AraC is enhanced or when there is excessive conversion of AraC to inactive AraU due to high levels of CDD [12]. Although multidrug resistance (MDR1) gene and multidrug resistance-associated protein (MRP) gene are commonly involved in drug resistance, both the genes have not been observed in AraC resistant cells [13]. Various studies have been performed to elucidate the mechanism of AraC resistance, but it still remains unexplained.

Therefore, the present study aimed at investigating the expression of proteins associated with the acquisition of resistance to AraC. The expression of the proteins was examined using two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) in AraC-sensitive (K562S) and AraC-resistant (K562AC) cell lines derived from K562.

Materials and Methods

Cell Culture

Human leukemia cell lines, K562S and K562AC, were obtained from the School of Medicine, Tohoku University. The resistant clones, termed K562/AC, were established and grown for 3 months, during which they were continuously exposed to increasing concentrations of Ara-C up to 50 mM, and were maintained in medium containing Ara-C. K562AC cells were established before, as described by Funato et al [13]. K562S cells were confirmed to be sensitive to AraC and K562AC cells were confirmed to be resistant to Ara-C (Nihon Shinyaku Co., Japan). The cells were grown in RPMI-1640 medium (Nissui Pharmaceutical Co., Japan) with 10% fetal calf serum (Cellec, OH, USA) at 37°C in 5% CO₂. Doxorubicin resistant derivative of K562 cell line was obtained from JCRB Japan, and maintained in medium containing Doxorubicin (Wako Co., Japan).

Cell Viability Assay

Cell viability was determined by the MTT assay method, using CellTiter 96 AQueous Cell Proliferation Assay (Promega, Madison, USA). A total of 10⁴ cell lines were plated in each well of a 48-well tissue culture plate. At the indicated times, cell viability was

measured. Untreated cells served as control. The viability of treated cells was calculated as a percentage of viability relative to the untreated control cells.

2D-DIGE

Cell pellets were dissolved in lysis buffer (7M urea, 2 M thiourea, 1% Triton X-100, 4% CHAPS, and 10 mM Tris). Cell lysates were labeled with Cy2, Cy3, and Cy5, according to the protocols described in the Ettan DIGE user manual (18-1164-40 Edition AA, GE Healthcare). The 2 Dimensional Fluorescence Difference Gel Electrophoresis (DIGE) was performed according to the method previously reported [14] with some modifications. IPG strips (24 cm, pH 3–10 nonlinear, GE) and Ettan IPGphor System (GE Healthcare, Uppsala, Sweden) were used for the first-dimension IEF. After 2-DE, gels were scanned on Typhoon 9410 scanner (GE Healthcare, Uppsala, Sweden) using the Ettan DALT gel alignment guides.

Gel Imaging and Statistical Analysis

Gel images were cropped using ImageQuant TL 2003 (GE Healthcare, Uppsala, Sweden) and spot detection was performed with DeCyder 6.5 DIA (Difference In-gel Analysis) software (GE Healthcare, Uppsala, Sweden). The gel images were matched using DeCyder 6.5 BVA (Biological Variation Analysis) software (GE Healthcare, Uppsala, Sweden). Statistical analysis was performed using paired Student's t-test with

false discovery rate correction using DeCyder 6.5 BVA. A $p < 0.05$ was considered statistically significant.

For protein identification, individual spots were digested with trypsin. Molecular mass analyses of triptic peptides were performed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) using an ultraflex TOF/TOF (Bruker Daltonics). Proteins were identified by comparison between the molecular weights determined by MALDI-TOF/MS and theoretical peptide masses from the proteins registered in NCBItr [15].

RNA Extraction and semi-quantitative analysis of RT-PCR products

Total RNA was isolated from cell lines with Isogen (Nippon Gene, Tokyo, Japan) and the RNA concentration was determined spectrophotometrically at 260 nm. Reverse transcription-PCR was performed using ReverTra Ace qPCR RT Kit (Toyobo, Tokyo, Japan). The following PCR primers were synthesized:

Vimentin forward: 5'-TGTTGACAATGCGTCTCTGGCAC-3', reverse:
5'-ACTCCAGTCCGAACCTTTGTAGG-3',

ALDH1A2 forward: 5'-AGTGTGGAGAAGGATGGATG-3', reverse:
5'-AGGTGTCGTATCAGGTTTCAG-3',

GAPDH forward: 5'-GCCAAGGTCATCCATGACAAC-3', reverse:
5'-AGTGTAGCCCAAGATGCCCTT-3'. Semi-quantitative analysis of RT-PCR
products was performed by using NIH ImageJ 1.34s software and normalized to
GAPDH. Shown were the results of one of three experiments with similar results.

Western Blot

Cell extracts were prepared in lysis buffer [100 mM Tris-HCL (pH 8.0), 100 mM NaCl, 0.5% NP40, and 0.2 mM phenylmethanesulfonyl fluoride], and centrifuged for five minutes at 1800 x g at 4°C. The supernatant was collected for protein analysis. Proteins in the supernatant were lysed with SDS-PAGE sample buffer, sonicated, and boiled. The protein samples were then separated by one-dimensional SDS-PAGE, transferred onto a PVDF membrane, and blotted with the appropriate antibodies. The immunoblot signals were normalized by the corresponding β -actin signal. Antibodies used for Western blot analysis were anti-vimentin polyclonal antibody (pAb), anti-ALDH1A2 monoclonal antibody (mAb), and anti-GAPDH pAb. Anti-vimentin pAb and anti-ALDH1A2 mAb were obtained from Abcam (Cambridge, UK) and anti-GAPDH pAb was obtained from Santa Cruz Biotechnology (CA, USA).

ALDH1A2 constructs and gene transfection.

We used RT-PCR to synthesize the full length cDNA for human ALDH1A2 (NM_170696). Total RNA was extracted from K562AC cells and used for the synthesis of the cDNAs. Full-length human ALDH1A2 cDNAs were subcloned into the N-terminus venus-tagged CAGGS expression vector. ALDH1A2 gene transfection to K562S cells was performed using Lipofectamine Plus (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. To establish stable transfectants, transfected cells were selected by the medium containing 500 μ g/ml G418, and clones derived from single cells were picked up.

RNA Interference Experiments Using Small Interfering RNAs (siRNAs)

ALDH1A2 siRNA-1 (s16907) was purchased from Ambion (Life Technologies, Japan). K562AC cells were transfected with siRNA using Lipofectamine Reagent (Life Technologies, Japan). As a negative control, a scrambled oligonucleotide, Negative control #2 (Life Technologies, Japan), was used. At 48 h after transfection, cells were treated with AraC for 24 h. Cell survival was determined using MTT assay, as described above.

Histopathology

Smear bone marrow slides were fixed with 100% methanol. The slide was treated with 0.3% hydrogen peroxide to block nonspecific staining. The primary antibody used

was rabbit anti-ALDH1A2 polyclonal antibody (Abcam, Cambridge, UK). The slide was incubated overnight with the antibody at 4°C. After washing in 50 mM Tris-buffered saline, a refined avidin-biotin technique in which a biotinylated secondary antibody reacts with several peroxidase-conjugated streptavidin molecules was employed for amplification using a DAKO LSAB+/HRP kit (Dako, Tokyo, Japan). Diaminobenzidine tetrahydrochloride (DAB) was used for the visualization of immunoreactive cells. The nuclei of cells were counterstained with hematoxylin. The total number of cells was counted; it was checked to determine whether the cells were positive or not. The ratio of positive cells to total cells was used to estimate the rate of ALDH1A2 positive cells.

Clinical Case

Peripheral blood was drawn from five patients with AraC resistance. The diagnosis of leukemia was based on clinical features and hematological characteristics. Ara-C resistance was clinically defined as follows: The case could not achieve complete remission (CR) by one or two courses of induction chemotherapy consisting of 12 mg/m² idarubicin [IDA] on days 1-3 and 100 mg/m² Ara-C on days 1-7 and salvage chemotherapy with high-dose Ara-C (2000 mg/m² Ara-C every 12 hours on days 1-5). CR was defined according to Cheson's criteria [16]. Informed consent was obtained

according to the Declaration of Helsinki. The study protocol comprised a retrospective case series design that was approved by the institutional review board at Gifu university hospital. Supplement Table 1 shows the main clinical findings of all the patients studied.

Statistical Analysis

The results depicted in Fig. 3, Fig. 4 and Fig. 5 are expressed as means \pm SD. Statistically significant differences ($p < 0.05$) between two groups were determined using Student's t-test.

Results

The protein profiles by 2D-DIGE

Protein expression in K562S and K562AC cells was compared using 2D-DIGE. AraC-resistance in K562 cells was established before, as described previously [13]. To confirm the resistance or sensitivity to AraC in K562S and K562AC cells, the viability of both cells after AraC treatment was examined using the MTT assay. After AraC treatment, there was no growth of K562S cells, while the proliferation of K562AC cells was unaffected. (Supplement Fig. 1).

In order to identify the proteins involved in the acquisition of AraC resistance, the differences in protein expression in K562S and K562A were examined by DIGE and mass spectrometry. The total number of differentially expressed proteins identified in the two cell lines was 2073. Of the proteins identified, the differences in expression of 15 proteins were found to be statistically significant (Fig. 1). These proteins were identified by mass spectrometry. Table 1 shows a list of the differentially expressed proteins with their protein accession numbers, calculated fold changes, and p values. The 15 proteins were assigned to four categories based on their function, namely macromolecular metabolism (amino acid and sugar metabolism), molecular cytoskeleton reorganization, signal transduction, and oxidative stress.

To validate the differential protein expression, RT-PCR and western blotting were performed for two proteins with the highest differential rate. The two proteins were found to be ALDH1A2 and vimentin (Fig. 2).

Effects of ALDH1A2 on AraC resistance

It has recently been reported that the amount of expressed ALDH isoform correlates with drug resistance [17] [18]. And, some reports showed that retinoic acid inhibited the vimentin expression [19]. We examined the expression of vimentin in K562S treated with retinoic acid. The retinoic acid treatment decreased the vimentin expression (data not shown). This result suggested that vimentin expression was controlled by ALDH1A2 expression. Thus, ALDH2 expression was considered to be more important to acquire the resistance than vimentin expression. Therefore, the effect of ALDH1A2 on AraC resistance in K562AC cells was investigated here. This was done by knocking down ALDH1A2 in K562AC cells using siRNA. ALDH1A2 knock down (KD) was confirmed using RT-PCR and western blotting (Fig. 3A). Cell viability was compared between the ALDH1A2 KD cells treated with and without AraC. The viability of ALDH1A2 KD cells was found to be reduced by AraC treatment. This shows that ALDH1A2 KD induces sensitivity to AraC in K562AC cells (Fig. 3B). To confirm the effect of ALDH1A2 on AraC resistance, the ALDH1A2 gene was

transfected to K562AS cells. The cells with this transfection acquired the resistance to AraC (Fig. 4).

Immunohistochemical analysis of ALDH1A2 in AraC-Resistant Patients

An in vitro study has suggested that ALDH1A2 may be associated with AraC resistance. Therefore, the expression of ALDH1A2 in the peripheral blood of AraC-resistant patients was examined using immunohistochemical analysis. ALDH1A2 expression in AraC-resistant patients was compared before and after the acquisition of resistance. Fig. 5 shows that ALDH1A2 expression was high in patients with AraC resistance and that all patients had increased ALDH1A2 expression after the acquisition of AraC resistance.

Discussion

The present study reveals that 15 proteins, including ALDH1A2, present in K562AC cells are significantly down- or up-regulated. ALDH1A2 belongs to the aldehyde dehydrogenase (ALDH) family. Twelve ALDH genes have been identified in humans. These genes, located on different chromosomes, encode a group of enzymes which oxidize a variety of aliphatic and aromatic aldehydes [20, 21]. Many studies have shown that some ALDH family members play an important role in drug resistance [17, 18]. ALDH3A1 (a member of the ALDH family) expression in tumor cells has been shown to induce resistance to drug toxicity, resulting in a poor response to anti-tumor drugs. As previously reported, ALDH3A1 is associated with resistance to etoposide and Mitomycin C [18]. It is also known that transfection with rat ALDH3 confers oxazaphosphorine-specific resistance on human MCF-7 cells. The resistance is also known to be reversed upon pretreatment with diethylaminobenzaldehyde, an ALDH3 inhibitor [22]. It has also been previously reported that overexpression of ALDH1A2 induces drug resistance [17].

ALDH1A2 is an enzyme that catalyzes the synthesis of retinoic acid from retinaldehyde. Retinoic acid is an active metabolite of vitamin A and regulates a wide range of biological processes including cell proliferation, differentiation, and

morphogenesis [23]. Recently, Moreb et al showed that the overexpression of both ALDH1A2 and ALDH2 results in increased relative resistance to both 4-Hydroperoxycyclophosphamide (4-HC) and doxorubicin, but resistant mechanisms did not revealed [17]. We also examined the protein expression pattern in K562 doxorubicin resistant cells using 2D-DIGE. 16 proteins were remarkably altered between K562 doxorubicin and K562S (Supplemental figure. 2). Our results showed that ALDH1A2 expression did not altered in K562 cell with doxorubicin resistance. The identified proteins were different between AraC resistance cells and doxorubicin resistance cells. These protein profiles revealed the participation of different proteins in the resistant mechanism of each two drugs.

The results of the present study using K562AC cells suggest that the expression of ALDH1A2 increases in AraC-resistant cells. Furthermore, ALDH1A2 KD is found to induce sensitivity to AraC treatment in K562AC cells, and ALDH1A2 overexpression on K562S cells induced the AraC resistance. It is speculated that ALDH1A2 expression in K562AC cells induces the accumulation of RA in the cytosolic space. Retinoic acid accumulation may be involved in the anti-apoptotic property. Kitamura et al. have shown that retinoic acid plays a role in the signal transduction pathways involved in hydrogen peroxide (H₂O₂)-induced apoptosis [24]. H₂O₂ induces apoptosis in mesangial

cells via activation of c-Jun-N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK). ERK and JNK induce accumulation and activation of activator protein-1 (AP-1), causing apoptosis. RA inhibits the apoptotic process by suppressing AP-1. This is because retinoic acid attenuates the expression of c-Fos/c-Jun and activation of JNK, at least in part, via induction of the dual-specificity protein phosphatase, MAP kinase phosphatase 1 (MKP-1) [25]. These findings suggest that ALDH1A2 may contribute to protection of drug-resistant cells from the apoptotic signaling by AraC in K562 cells.

Further studies are still needed to verify the possible mechanisms for AraC resistance. However, the results of the present study provide useful insights into the mechanisms of drug resistance and allow us to assume that various molecules participate in the mechanism. It is difficult to elucidate the actual mechanism of drug resistance because of the up-regulation of only ALDH1A2, although it is known that ALDH1A2 plays an important role. Studying the interaction between ALDH1A2 and other molecules may be useful for the elucidation of the mechanism of AraC resistance.

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Conflict of Interest statement

The authors declare that there are no conflicts of interest.

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Figure captions

Figure 1.

2D-DIGE gel images of K562S labeled with Cy5 and K562AC labeled with Cy3. (A) Spots of proteins differentially expressed ($p < 0.05$, fold change ≥ 2.0) were selected for identification. (B) Spots 4, 5 and 6, examples evaluated using Decyder. The top panel is the partial view. The middle panel is the 3D image. The bottom panel analyzed to each spot about relative protein abundance (Standard, K562 and K562AC).

Figure 2.

RT-PCR and western blotting analysis of vimentin, ALDH1A2, and GAPDH in K562S and K562AC.

To validate the differing expressions of proteins in K562S and K562AC, expression of both ALDH1A2 and vimentin genes was observed in both cell lines. (A) RT-PCR showing the mRNA expressions of ALDH1A2 and vimentin in K562S and K562AC. (B) Western blots showing the expression level of ALDH1A2 and vimentin in K562S and K562AC.

Figure 3.

Effects of AraC treatment in K562AC with ALDH1A2 KD

(A) ALDH1A2 KD in K562AC examined by RT-PCR and western blot. (B) Proliferation of K562AC with ALDH1A2 KD after AraC treatment examined using the proliferation assay. ALDH1A2 KD induced sensitivity to AraC. Data are shown as the mean \pm SD of three independent experiments. * $p < 0.05$.

Figure 4.

Effects of ALDH1A2 overexpression in K562S.

(A) ALDH1A2 overexpression in K562S confirmed by western blot. (B) Proliferation of K562S with ALDH1A2 overexpressed after AraC treatment examined using the proliferation assay. ALDH1A2 overexpression induced resistance to AraC. Data are shown as the mean \pm SD of three independent experiments. * $p < 0.001$.

Figure 5.

Immunohistochemical analysis of ALDH1A2 in AraC resistant patients.

(A) ALDH1A2 expression observed in the peripheral blood of Ara-C-resistant patients before and after the acquisition of AraC resistance. The results of one of the five analyses are shown because similar results were obtained in the five analyses (a, c: lower magnification, b, d: higher magnification). (B) Changes in ALDH1A2 expression

in leukemia cells before and after the acquisition of AraC resistance. The rate of positive cells in all AraC resistant patients is summarized. * $p < 0.05$.

Supplement Figure 1.

Viability of K562S and K562AC cells after AraC treatment.

K562S and K562AC cells were treated in the presence or absence of AraC. After 24 h, 48 h, and 72 h of AraC treatment, proliferation was determined by the cell proliferation assay. The results are expressed as the mean \pm SD of three independent experiments.

Supplement Figure 2.

2D-DIGE gel images of K562S labeled with Cy5 and K562DOX labeled with Cy3. (A) Spots of proteins differentially expressed ($p < 0.05$, fold change ≥ 2.0) were selected for identification.

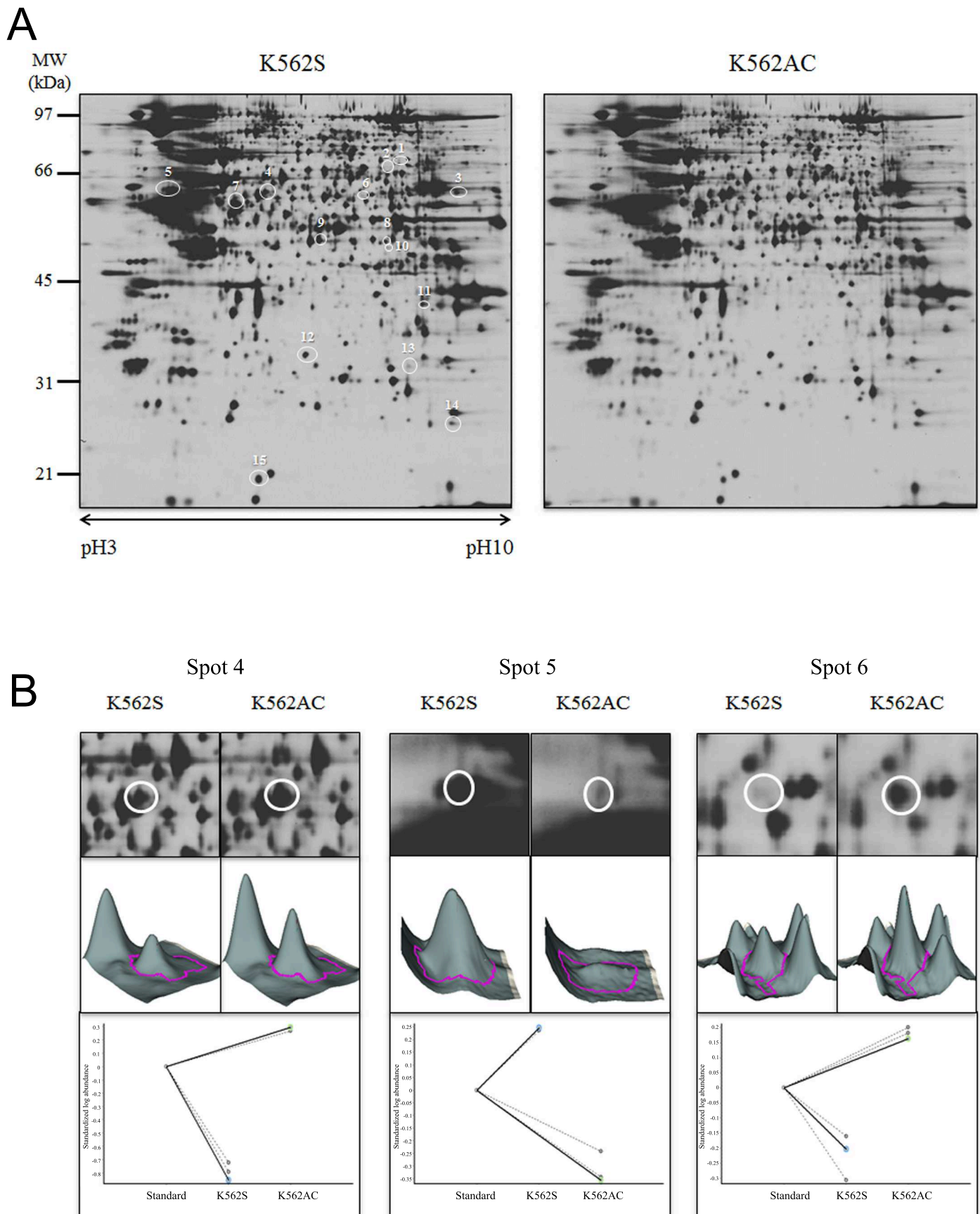


Figure 1. Kawasoe et al

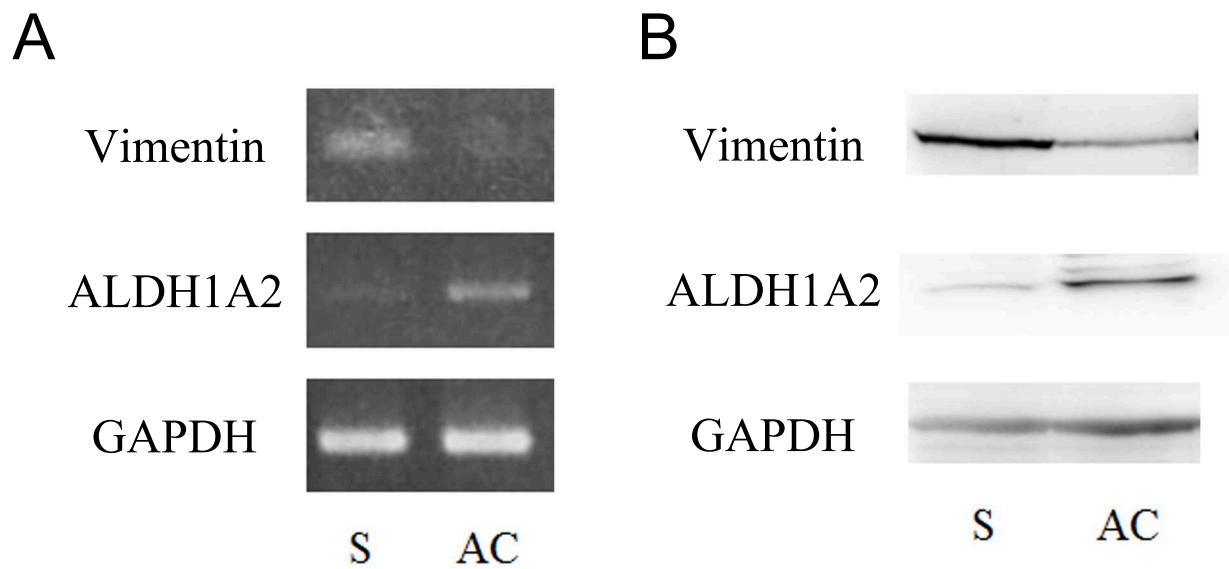
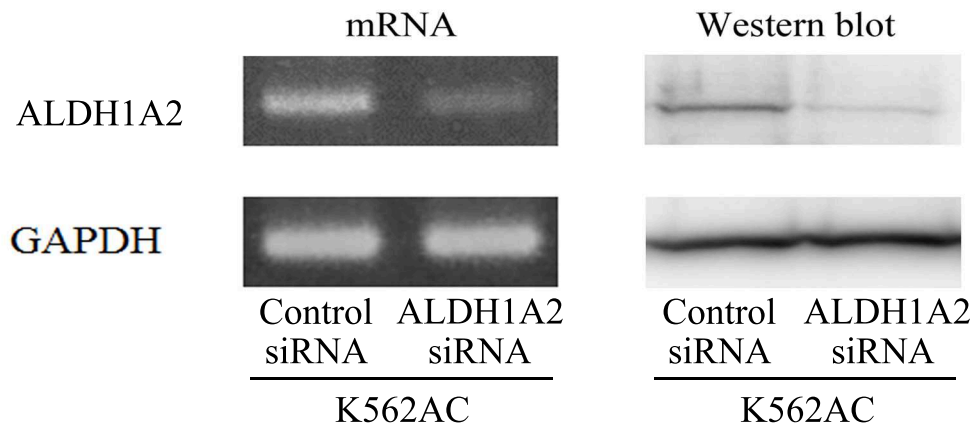


Figure 2. Kawasoe et al

A



B

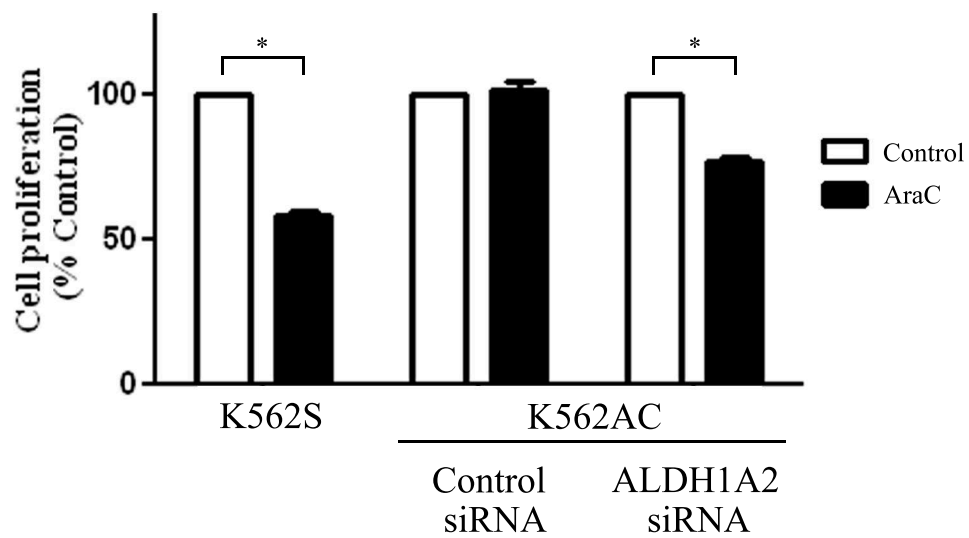
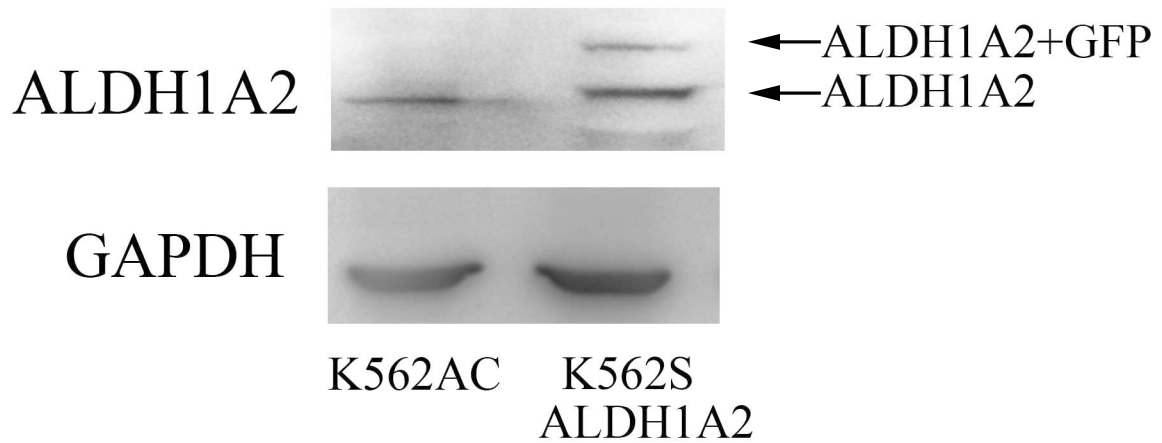


Figure 3. Kawasoe et al

A



B

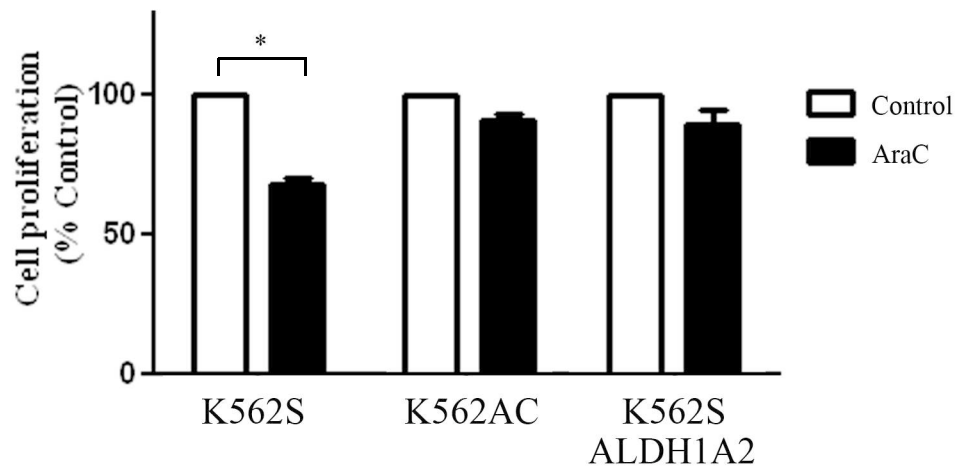
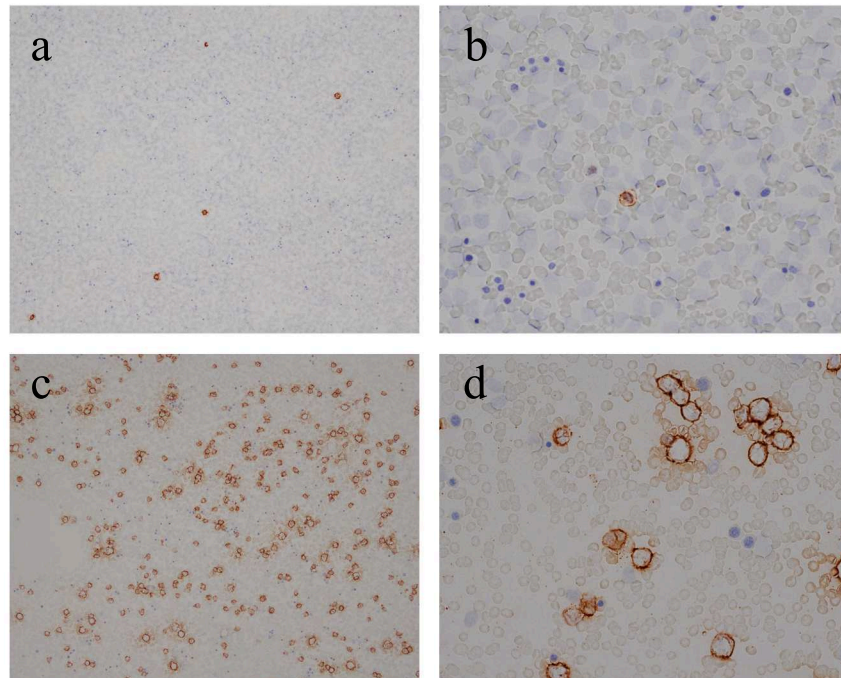


Figure 4. Kawasoe et al

Resistance



B

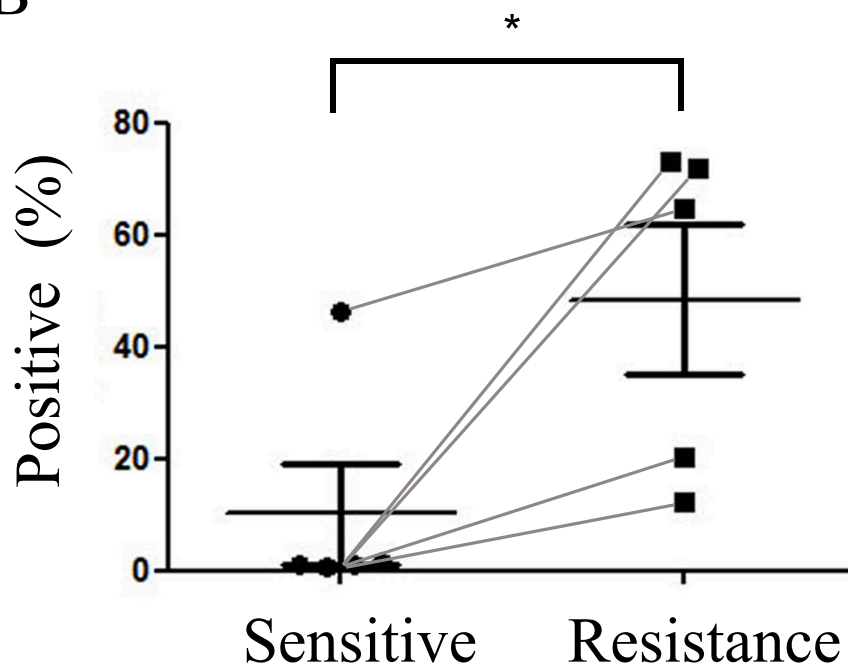
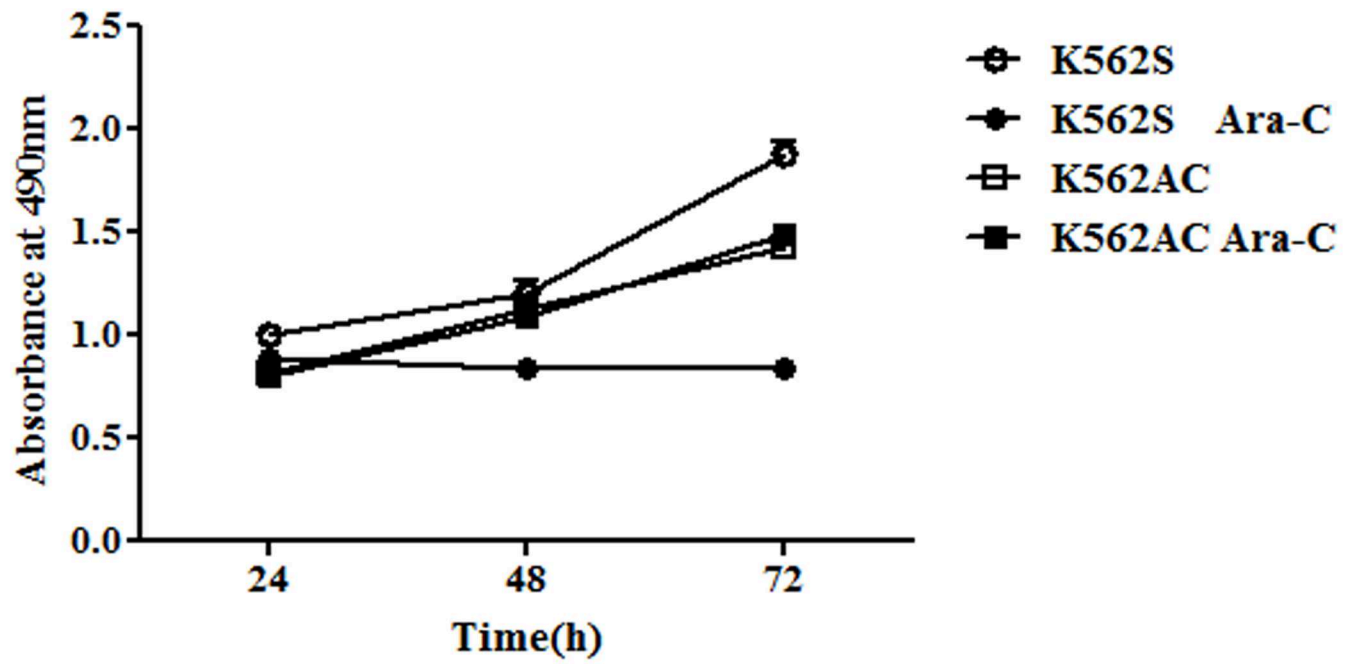


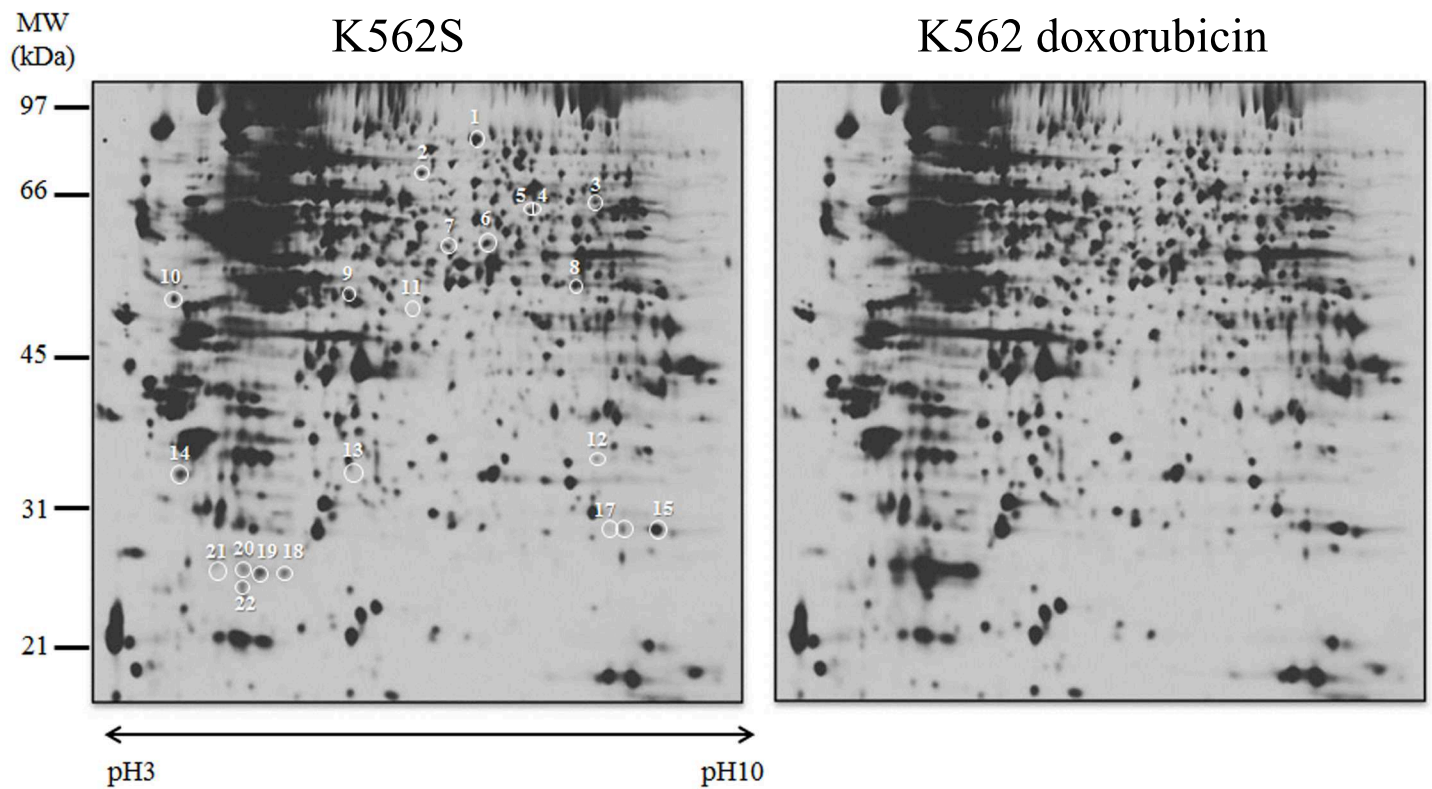
Figure 5. Kawasoe et al

Table 1.

Spot no.	Protein name	ID (NCBI)	p-value	Fold change
1	Phosphoenolpyruvate carboxykinase	gi290457671	0.0016	2.18
2	Syntaxin-binding protein 1	gi50403646	0.0016	2.53
3	Glucose-6-phosphate isomerase	gi17380385	0.0053	-2.28
4	Aldehyde dehydrogenase 1 family, member A2	gi25777724	0.00088	11.48
5	Vimentin	gi55977767	0.0016	-3.59
6	D-3-phosphoglycerate dehydrogenase	gi21264510	0.0040	2.52
7	Cytokeratin-8	gi90110027	0.00097	2.73
8	hnRNP D0	gi13124489	0.0018	-2.00
9	Ornithine aminotransferase	gi129018	0.00076	-2.39
10	Medium-chain specific acyl-CoA dehydrogenase	gi113017	0.0023	2.63
11	Annexin A2	gi113950	0.0049	2.00
12	Glutathione S-transferase omega-1	gi6016173	0.00088	2.11
13	Carbonic anhydrase 2	gi115456)	0.0200	-2.63
	Phosphoglycerate mutase 1	gi130348		
14	Transgelin-2	gi586000	0.0025	2.37
15	Superoxide dismutase [Cu-Zn]	gi134611	0.0031	-2.01



Supplement Figure 1. Kawasoe et al



Supplemental Figure 2. Kawasoe et al

Supplement Table 1			
Characteristics of the patients	Age	Sex	FAB subtype
Acute myelogenous leukemia	59	F	M2
	31	M	M4
	37	M	M4
	48	F	M5a
	53	M	M5a

Supplemental table 2.

Spot no.	Protein name	ID (NCBI)	p-value	Fold change
1	Ezrin	gi125987826	0.0640	3.19
2	Leukotriene A-4 hydrolase	gi126353	0.0041	2.33
3	Catalase	gi115702	0.0054	2.31
	Pyruvate kinase isozymes R/L	gi8247933		
4	Inosine-5'-monophosphate dehydrogenase 2	gi124419	0.0021	2.92
5	Glucose-6-phosphate 1-dehydrogenase	gi116242483	0.0048	2.19
6	Lupus La protein	gi125985	0.0030	2.24
7	Lupus La protein	gi125985	0.0025	2.15
8	Multifunctional protein ADE2	gi131628	0.0016	2.38
9	Serpin B9	gi1709896	0.0020	-2.39
10	Reticulocalbin-1	gi2493462	0.0038	-3.43
11	Macrophage-capping protein	gi313104088	0.0021	3.29
12	Phosphoglycerate mutase 1	gi130348	0.0021	-2.52
	Carbonic anhydrase 2	gi115456		
13	15-hydroxyprostaglandin dehydrogenase [NAD+]	gi129889	0.0050	5.61
	Putative ATP-dependent Clp protease proteolytic subunit	gi3023512		
14	Kinetochore protein Spc24	gi74751176	0.0025	-4.56
	Calbindin	gi115394		
15	Peroxiredoxin-1	gi548453	0.0021	2.30
16	Flavin reductase	gi1706870	0.0030	2.42
17	Flavin reductase	gi1706870	0.0024	2.60
18	Sorcin	gi267021	0.0048	4.60
19	Sorcin	gi267021	0.0066	2.12
20	Sorcin	gi267021	0.0018	6.05
21	Sorcin	gi267021	0.0025	2.81
22	Sorcin	gi267021	0.0021	3.72